1 **Variations in methods for quantification of crude ash in animal feeds**

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20 **Abstract**

21 **Background:** Crude ash is categorized as an empirical method playing an important role

22 in the nutritional interpretation of animal feeds, allowing indirect estimation of total

23 organic matter.

24 **Objective:** Our objective was to evaluate variations in laboratory procedures for crude

25 ash quantification regarding physical parameters (i.e., time, temperature) and ashing aids

26 and their influences on crude ash, repeatability, and discrimination power among feeds.

27 **Method:** The "control" method was based on a simple ignition time of 3 h at 550°C. The

28 variations are briefly described: increasing ashing time to 6 h; increasing temperature to

29 600°C; and using two 3-h ignition cycles at 550°C with ashing aids inclusion between

30 them: fresh air supply, fresh air supply plus distillate water, and fresh air supply plus

31 hydrogen peroxide. A color evaluation was also performed using a colorimetric

32 technique. Twenty four study materials from eight different feed types were evaluated.

33 **Results:** The crude ash results differed among the method variations, but a consistent

34 decrease in the estimates was observed when liquid aids were applied, which also

35 improved repeatability. Ash residues did not present a consistent color pattern among

36 methods, but the residues were darker when the control method was applied.

37 **Conclusions:** The method of obtaining ash residues in animal feeds based on the binomial

38 550°C × 3 h does not have enough robustness and may overestimate crude ash in some

39 feeds. Adjustments in either ignition time or temperature might improve crude ash test

40 results, but the best test results are obtained using liquid ashing aids between two ignition

41 cycles.

42 **Highlights:** The recommended method is based on the use of 550°C and two 3-h ignition

43 cycles with water added to the ash residue between cycles.

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44 **Introduction**

45   The terms crude ash or mineral matter refer to the inorganic residue after complete

46 oxidation of organic matter (OM) (1, 2). However, some authors have stated that this

47 residue should be more properly termed as a residue on ignition (3) when oxidation is

48 mostly provided by burning. Generally, the ashing procedures comprise vaporization of

49 water and volatile compounds, and conversion of minerals into silicates, phosphates,

50 oxides, sulfates, and chlorides, in addition to oxidizing organic substances (2).

51   Crude ash plays an important role in the nutritional interpretation of animal feeds

52 by allowing indirect estimation of total OM, which encompasses all potential energy-

53 producing compounds (4). Moreover, ash contents are also mandatory to estimate feed

54 components quantified by difference, such as non-fiber carbohydrates and nitrogen-free

55 extract (5, 6). Crude ash estimates have been incorporated as an important input into

56 summative systems for the estimation of energy contents in animal diets (7-9). Actually,

57 any bias on the crude ash estimates might decrease the accuracy of nutritional evaluation

58 of animal feeds, which in turn may compromise production and culminate in economic

59 losses (3) due to inadequate diet formulation.

60   The standard method used to estimate % crude ash in animal feeds was first

61 described in the early 20th century (AOAC method 942.05; 10, 11) and is still used

62 worldwide as is or with some minor modifications. Briefly, a 2-g test portion is ignited at

63 600°C for 2 h and crude ash is reported as the residue on ignition (3).

64   Nevertheless, the crude ash in animal feeds could be categorized as an empirical

65 or type I method. Thus, it would be an analytical entity that determines a value that can

66 only be achieved in terms of the method *per se* (12). In this sense, there are no primary

67 reference standards that simulate the complex associations between organic and inorganic

68 compounds, such as those observed in feed materials. Therefore, the method itself cannot

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69 be validated for accuracy in determining the "true" value for the constituent. To minimize

70 systematic errors (i.e., bias) among laboratories, empirical methods must be followed

71 exactly as described in the standard manuals. Even minor variations in methodology

72 might result in the measurement of a different constituent (13) and compromise feed

73 interpretations and comparisons among feeds, laboratories, and analysts.

74   The ashing procedure can be described as the submission of a test portion to a

75 physical binomial based on temperature and time. Despite being originally based on using

76 600ºC, lower ignition temperatures have been suggested for the official AOAC method

77 since the 1940s (10, 11, 14). Excessive temperatures have been associated with systematic

78 bias caused by the volatilization of several minerals (3, 5, 15, 16). Thiex et al. (3) revisited

79 the official AOAC method 942.05 and suggested temperature adequacy down to 550ºC.

80 Some standards for feed analysis have followed a similar pattern of temperature

81 adequacy, such as in ISO method 5984:2002 (17), European Commission (18), or the

82 latest edition of the Brazilian standards for animal feed analyses (method M-001/2; 6).

83   On the other hand, the second variable of the physical binomial applied to crude

84 ash quantification seems to have a more controversial pattern in the literature.

85 Recommendations for ashing time can range from 1 h (1) to overnight (16, 19) or until a

86 constant weight of residue on ignition is achieved (20). It is known that oxidation power

87 into the muffle furnace results from a balance between temperature and time (21). Thus,

88 in a logical reasoning, the higher the temperature, the shorter the ignition time, and vice-

89 versa. However, most methods seem to use modal times ranging from 2 to 3 hours as they

90 are based on temperatures between 550-600ºC. In general, the use of longer ignition times

91 seems to be an attempt to either avoid or minimize the contamination of residue by a part

92 of OM that is possibly more refractory to oxidation. The influence of that refractory part

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93   has been associated with dark/brown colorations of the residue on ignition (3) even after

94   many hours of ashing.

95   In this sense, the introduction of ashing aids as a third element contributing to OM

96   oxidation (i.e., besides temperature and time) has been suggested. Accordingly, Thiex et

97   al. (3) recommended a modification to the AOAC official method 942.05, which should

98   encompass two 3-h ignition cycles at 550ºC. The cycles are intercalated by opening the

99   furnace door to guarantee a fresh air supply. In this case, the fresh air would serve as an

100 oxygen supplier and would improve the oxidation of refractory organics. On the other

101 hand, some authors have claimed that the dry ashing process may produce a heavy layer

102 on top of the ash residue, which could hinder its complete combustion and overestimate

103 ash content. In this case, a small amount of water could be poured over the ash residue

104 between two ignition cycles to break up that layer (2, 3). Moreover, some variations in

105 dry ashing methods have been based on the use of chemical substances as ashing aids (3,

106 18, 22, 23), probably relying on the release of free radicals to speed up oxidation.

107   However, despite of all the current theoretical knowledge on the dry ashing

108 process, the recommendations regarding laboratory procedures are still highly variable

109 (1, 2, 3, 6, 16, 18). Possibly, a "perfect" standard procedure cannot be achieved for all

110 feed materials (3). Notwithstanding, the efficiency of variations in the procedures must

111 be verified to assure adequate levels of method robustness while keeping an optimal

112 ability to discriminate feed materials regarding their different chemical characteristics.

113   Thus, our objective was to evaluate some variations in laboratory procedures for

114 crude ash quantification regarding physical parameters (i.e., time, temperature) and

115 ashing aids and their influences on crude ash estimates, repeatability, and discrimination

116 power among feed types.

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118 **Experimental**

119 *Location and study materials*

120   All analyses were performed at the Animal Nutrition Laboratory of the Animal

121 Science Department of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

122   Eight different feed types were chosen aiming to compose a representative set

123 regarding diets offered to beef and dairy cattle: corn silage, fresh sugarcane, sugarcane

124 silage, Tifton-85 hay, soybean meal, corn grain, wheat bran, and dried distillers' grains

125 (DDG). For each feed type, three different unique materials (i.e., field replicates or

126 primary samples) were obtained from feed industries and farms located in Minas Gerais

127 State, Brazil. Therefore, our analytical set encompassed 24 study materials.

128   The high-moisture feeds (silages and fresh sugarcane) were oven-dried (55°C).

129 Then, all dry study materials were ground in a knife mill (TE-680, Tecnal, Piracicaba,

130 São Paulo, Brazil) to pass through a 1-mm screen sieve. The 24 study materials were then

131 analyzed in triplicate for dry matter (DM) content (dried overnight at 105°C, method G-

132 003/1; 6).

133 *Crude ash methods*

134   The "control" procedure herein was based on the official method of the Brazilian

135 National Institute of Science and Technology in Animal Science (INCT-CA; method M-

136 001/2; 6). Briefly:

137   **(a)** Weigh 2.0 g as-is of the test portion into the crucible, recording the weight of

138   crucible and test portion nearest 0.1 mg;

139   **(b)** Add the crucibles into the muffle furnace;

140   **(c)** Ignite in a furnace at 550°C for 3 h. The furnace must be adjusted to reach ignition

141   temperature within 60 min. The ignition time starts counting after ignition

142   temperature is achieved;

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143   **(d)** Allow the furnace to cool below 200°C, yet above 150°C. Then transfer crucibles

144   to a desiccator; and

145   **(e)** Cool to room temperature and weigh, recording the weight to the nearest 0.1 mg.

146   For all procedures, the crucibles (5-cm diameter and 30-mL volume) were

147   previously washed in running water, ashed for 3 h at 550°C, and handled and weighed as

148   described above. The same digital muffle furnace was used for all procedures (Fornos

149   Magnus, Belo Horizonte, Minas Gerais, Brazil).

150   The following variations on the basic method were evaluated:

151   1. Increased ignition temperature: all procedures were performed as previously

152   described for the control, but the temperature was increased to 600°C (2, 10);

153   2. Increased ashing time: all procedures were performed as previously described for

154   the control, but ashing time was increased to 6 h. This specific time was chosen

155   to allow a direct comparison with method variations that included ashing aids;

156   3. Using fresh air as an ashing aid: the procedures were adapted from Thiex et al.

157   (3). The following modifications in the control were added:

158   **(d)** Allow the furnace to cool below 200°C° and open the door to ensure a fresh

159   air supply (1-2 min);

160   **(e)** Reignite in a furnace at 550°C for 3 h. The ignition time starts counting after

161   ignition temperature is achieved;

162   **(f)** Allow the furnace to cool below 200°C, yet above 150°C. Then transfer

163   crucibles to a desiccator; and

164   **(g)** Cool to room temperature and weigh, recording the weight to the nearest 0.1

165   mg;

166   4. Using fresh air and water as ashing aids: the procedures were adapted from Thiex

167   et al. (3) and, besides fresh air supply, it aims at breaking the superficial layer of

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168   residue on ignition and allowing more efficient ashing of the bottom layer in the

169   second ignition cycle. The modifications of procedures compared to the control

170   were:

171   **(d)** Allow the furnace to cool below 100°C and open the door;

172   **(e)** Carefully add a few mL of distilled water to the residue to break it up;

173   **(f)** Reignite in a furnace at 550°C for 3 h. The ignition time starts counting after

174   ignition temperature is achieved;

175   **(f)** Allow the furnace to cool below 200°C, yet above 150°C. Then transfer

176   crucibles to a desiccator; and

177   **(g)** Cool to room temperature and weigh, recording the weight to the nearest 0.1

178   mg;

179   5. Using fresh air and hydrogen peroxide as ashing aids: the procedures were the

180   same as described in the latter variation, except that water was replaced by

181   hydrogen peroxide PA (35% or 130 vol.). In this case, besides breaking up the

182   upper layer, we hypothesized the hydrogen peroxide decomposition releases free

183   radicals, which could speed up the oxidation of the residual organic matter.

184   Each ashing run contained all 24 study materials. We performed three ashing runs

185 for each method, totaling the evaluation of 432 aliquots (i.e., test portions) (n = 3).

186   Following the recommendations of Thiex et al. (3), we also performed a color

187 evaluation of residues on ignition. Due to the small masses, the residues from the three

188 replicates were pooled and scored for color pattern (L* for lightness, a* for redness, and

189 b* for yellowness), using a Hunter MiniScan EZ colorimeter (4500 L; Hunter Associates

190 Laboratory Inc., Reston, Virginia, USA). Those coordinates were then converted into

191 RGB (i.e., red, green, and blue) coordinates using Coloroid Professional Color Plan

192 Designer software. After that, RGB coordinates were organized in an Excel plan in which

193   each cell was filled with the correspondent solid color. For some materials, the mass of

194   residues on ignition was not sufficient to allow the color evaluation.

195   *Calculations and statistical analysis*

196   The calculation of crude ash was performed according to the following equation:

197     $$\%CA_{DM} = \frac{R - T}{W \times DM} \times 100 \tag{1},$$

198   where $\%CA_{DM}$ is the crude ash as a percentage of dry matter, R is the weight of crucible

199   + residue on ignition (g), T is the tare (empty) weight of crucible (g), W is the weight of

200   test portion (g as-is), and DM is the dry matter content of the sample (g/g).

201   The crude ash results were analyzed according to the model:

202     $Y_{ijkl} = \mu + F_i + S_{(i)j} + M_k + FM_{ik} + \varepsilon_{ijkl}$

203   (2),

204   where Y_{ijkl} is the crude ash obtained in the test portion l taken from study material j of

205   feed type i and evaluated through method k, μ is the general constant, F_i is the fixed effect

206   of feed type i, $S_{(i)j}$ is the random effect of study material j nested to feed i assumed to be

207   NIID (0, $\sigma_{S/F}^2$), M_k is the fixed effect of method k, FM_{ik} is the fixed effect of interaction

208   between feed type i and method k, and ε_{ijkl} is the random error assumed to be NIID (0,

209   σ_{ε}^2).

210   When necessary, means were grouped using the Fisher's multiple comparison

211   procedure.

212   After the first analysis of variance, data was analyzed again in an independent way

213   for each method according to the model:

214     $Y_{ijk} = \mu + F_i + S_{(i)j} + \varepsilon_{ijk}$

215   (3),

216   where Y_{ijk} is the crude ash obtained in the test portion k taken from the study material j

217   of feed type i, μ is the general constant, F_i is the random effect of feed type i assumed to

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218 be NIID $(0, \sigma_F^2)$, $S_{(i)j}$ is the random effect of study material j nested to feed type i assumed

219 to be NIID $(0, \sigma_{S/F}^2)$, and ε_{ijk} is the random error assumed to be NIID $(0, \sigma_\varepsilon^2)$.

220 From the adjustment of the model (3), the following relative standard deviations

221 were estimated for each method variation:

222 $\text{r} = \dfrac{\sqrt{\hat{\sigma_\varepsilon^2}}}{\overline{Y}} \times 100$ 　　　　　　　　　　　(4),

223 $\text{RSD}_F = \dfrac{\sqrt{\hat{\sigma}_F^2}}{\overline{Y}} \times 100$ 　　　　　　　　　　　(5),

224 $\text{RSD}_S = \dfrac{\sqrt{\hat{\sigma}_{S/F}^2}}{\overline{Y}} \times 100$ 　　　　　　　　　　　(6),

225 where r is the repeatability (%), $\hat{\sigma}_\varepsilon^2$ is the estimate of error variance [(% DM)²], $\overline{Y}$ is the

226 average crude ash (% DM), RSD_F is the relative standard deviation among feed types

227 (%), $\hat{\sigma}_F^2$ is the estimate of the variance among feed types [(% DM)²], RSD_S is the relative

228 standard deviation among study materials (%), and $\hat{\sigma}_{S/F}^2$ is the estimate of the variance

229 among study materials within feed types [(% DM)²].

230 Although some reference values have been established regarding reproducibility

231 (24, 25), it has been difficult to define adequate limits for repeatability. We know that the

232 reproducibility evaluation cannot be performed in our work, as all procedures were

233 performed in one single laboratory. However, we adopted a more functional approach to

234 assess repeatability from the expected reproducibility value, which was calculated as:

235 $Re = 2 \times C^{-0.15}$ 　　　　　　　　　　　(7),

236 where Re is the expected reproducibility (%), and C is the average content of crude ash

237 (g/g DM).

238 According to Horwitz (24), repeatability should ordinarily be one-half to two-

239 thirds of reproducibility. From this, we assumed that the expected conventional limits for

240 repeatability could be established as:

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241 $$L_c = 0.50 \times Re \tag{8a},$$

242 $$U_c = 0.67 \times Re \tag{8b},$$

243 where L_c and U_c are the lower and upper limits for the expected conventional repeatability

244 (%).

245   Equation (8) is based on the assumption that reproducibility behaves exactly as

246 expected (Equation 7). However, in general, a method is considered reproducible if the

247 actual reproducibility falls between one-half and two-fold of expected reproducibility (24,

248 25). From this, the conventional limits for repeatability may be adjusted to a tolerable

249 range according to:

250 $$L_t = 0.50 \times 0.50 \times Re = 0.25 \times Re \tag{9a},$$

251 $$U_t = 0.67 \times 2.00 \times Re = 1.34 \times Re \tag{9b},$$

252 where L_t and U_t are the lower and upper limits for the expected tolerable repeatability

253 (%).

254   From equations (8) and (9) we were able to understand how adequate the method

255 variations behaved regarding precision/repeatability.

256   All statistical evaluations were performed using the GLIMMIX procedure of SAS

257 9.4. The variance components were estimated according to the restricted maximum

258 likelihood method. Statistical significances were declared at $P < 0.05$.

259

260 **Results**

261   There was an interaction ($P<0.01$) between feed types and methods on the crude

262 ash. The slicing of this effect indicated that for most feed types (corn grain, DDG, wheat

263 bran, grass hay, and fresh sugarcane) the methods did not affect ($P \geq 0.23$) the values of

264 residues on ignition (Table 1). However, differences among methods occurred ($P<0.01$)

265 for soybean meal, corn silage, and sugarcane silage.

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266   The comparisons among methods within each feed type were generally different

267 from each other and some overlaps were observed (Table 1). For soybean meal, the

268 general pattern indicated that just increasing either time or temperature was capable to

269 decrease (P<0.05) crude ash in comparison to the control method. However, a more

270 consistent decrease compared to the control (P<0.05) was verified when ashing aids were

271 applied. All ashing-aid variations clustered together (P>0.05). On the other hand, the

272 mean comparisons for corn silage indicated that increasing neither temperature nor ashing

273 time was sufficient (P>0.05) to decrease crude ash in comparison to the control. Effective

274 decreases were only obtained (P<0.05) when liquid ashing aids were used, which did not

275 differ from each other (P>0.05). The mean comparisons for sugarcane silage were very

276 uninformative and a clear pattern could not be extracted from them.

277   On average, the overall mean comparisons indicated that increasing either

278 temperature or ashing time caused a consistent decrease in crude ash when compared to

279 the control method (P>0.05, Table 1, Figure 1). In terms of ashing aids, the simple

280 introduction of fresh air was not enough to decrease (P>0.05) crude ash in comparison to

281 simply increasing the temperature or time. However, the use of liquid ashing aids caused

282 an additional decrease (P<0.05) in crude ash compared to the other methods.

283   The relative standard deviations among feed types and study materials behaved

284 similarly among methods (Table 2). On the other hand, repeatability was found within

285 the tolerable boundaries for all methods. However, when liquid ashing aids were added,

286 the repeatability was slightly improved.

287   The color evaluation was fully performed only for four feed types (Table 3). For

288 the others, we faced constraints to obtain residues masses in sufficient quantities to allow

289 an adequate color measurement by the colorimetric method. However, for the feed

290 materials we had, there was no consistent pattern among methods. However, one specific

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291 point in the pattern seemed consistent enough to draw some inference. The residues on

292 ignition were darker when the control method was applied, which is in line with the crude

293 ash results. No systematic differences in the color pattern were verified among the other

294 methods.

295

Discussion

297   The crude ash in feeds is assessed as the residue on ignition after a complete

298 organic matter decomposition in a muffle furnace using a time × temperature binomial.

299 Due to its empirical nature, ashing methods have been revised (3, 26) aiming at

300 minimizing their associated biases and seeking a balance between complete oxidation and

301 loss of volatile minerals. In general, biases might be generated from incomplete organic

302 matter decomposition, volatilization of some mineral compounds under specified

303 temperatures, or inappropriate method application (1, 3, 16, 26).

304   Despite being independent physical parameters, time and temperature do not act

305 independently, and interactions between them will define the amount of crude ash

306 obtained from a specific feed material (21). Once the adequate temperature is established,

307 adjustments on ignition time would provide some fine-tuning on the analytical entity's

308 estimates and vice-versa. On the other hand, temperatures below the minimum required

309 for adequate ignition of the organic matter may not be compensated by extending ignition

310 time. An equilibrium between the physical parameters of the process must be achieved to

311 allow an appropriate quantification of crude ash in feeds.

312   The results here confirmed that increases in either ignition time or temperature

313 led, on average, to decreases in the % crude ash. It brings evidence that the physical

314 binomial 550°C × 3 h is not the best option to quantify the crude ash in feed materials.

315 This was the only clear pattern pointed out by the colorimetric evaluation of residues on

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316   ignition. Some feed types were not sensitive to the variations in the physical parameters;

317   however, others were. Thus, to achieve a more adequate robustness, the chosen method

318   should focus on the feeds types that are particularly sensitive to the method.

319   An ideal ignition temperature should be as low as possible to reduce volatile

320   compound losses, yet high enough to ensure total carbon loss (23). A recurrent issue

321   associated with excessive temperatures is the loss of minerals through volatilization (26,

322   27). From this, it could be speculated that increasing the temperature to 600°C could have

323   decreased ash content due to increased volatilization. However, the decreased ash content

324   obtained by extending ashing time seems to allow a different explanation for that.

325   Considering that the simple extension of either time or temperature led to the same

326   average decrease in % crude ash, the most probable cause was an improvement in the

327   elimination of some refractory organic matter, rather than an increase in volatilization.

328   Once more, the inadequacy of the control procedure is evident.

329   However, despite the likely improvement aforementioned, the % crude ash was,

330   on average, further reduced with the use of liquid ashing aids. In this particular case, any

331   loss increased by volatilization has no physical or chemical reasons to occur. However,

332   the pattern obtained with the different ashing aids was not consistent among feed types.

333   Despite clustering along with the liquid ashing aids for soybean meal and

334   sugarcane silage, on average, the simple fresh air supply was not enough to reduce the

335   crude ash at the same levels observed when liquid aids were applied. It has been stated

336   that a fresh air supply between two ignition cycles could renew the oxygen supply inside

337   a muffle furnace. Consequently, it could improve the release of carbon that might remain

338   in the sample after the first ignition cycle (3, 28). However, such a pattern did not

339   correspond to what was observed here.

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340   Further improvements in crude ash estimates were obtained only when liquid

341   ashing aids were added between the two ignition cycles. During the ashing procedure, a

342   heavy layer might be formed on the top of the residue interfering with carbon release (2).

343   Then, adding liquid aids between ignition cycles might improve organic matter

344   decomposition by a mechanical act, crushing the crust eventually formed in the previous

345   ignition cycle and improving the degradation of refractory compounds in the second

346   ignition cycle (27). Hydrogen peroxide might accelerate organic matter combustion in

347   dry ashing methods (23). The decomposition of hydrogen peroxide basically occurs when

348   its highly unstable oxygen-oxygen bond is broken, which can release free radicals, such

349   as hydroxyl and hydroperoxyl (29). Those are highly reactive and may improve organic

350   matter decomposition. However, we did not observe differences between using water and

351   hydrogen peroxide as ashing aids, indicating that their effects were similar and most likely

352   associated with the physical breakdown of the heavy layer in the top of the ash residue,

353   allowing a better oxidation from material below the crust during the second ignition cycle.

354   Considering the similarity of both liquid ashing aids, water is recommended, mainly due

355   to its lower cost and ease of access.

356   Several authors have considered that complete ashing is reached when the heating

357   is continued until residue on ignition achieves a uniform color and is free from unburned

358   particles (30, 31). In this sense, the color should be as light as possible (i.e., white, light

359   gray; occasionally reddish or green). Accordingly, if the residue is dark or brown, it could

360   indicate an undesirable carbon presence (3, 28, 31, 32).

361   However, any visual color evaluation can be biased, as it depends on the subject's

362   judgment. There is no standard color chart available for color evaluations of ash residue,

363   which makes visual evaluations subjective and imprecise. Color scoring would be the

364   result of a light interaction between the object and the observer's eyes, and can also vary

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365 according to the ambient lighting (33). That is the reason why we decided to perform a

366 color evaluation using a colorimetric technique, which would avoid any subjectivity when

367 scoring the residue coloration. In general, the control method (i.e, 550°C × 3 h) presented

368 the darkest residues, but no clear pattern among feeds was observed for the other methods.

369 Despite confirming the inadequacy of the control method, the inconsistent pattern

370 among methods indicates that ash color seems to be more a characteristic of the feed

371 material itself rather than a completely useful information to evaluate the ashing quality.

372 Even though improvements in crude ash had been obtained using liquid ashing aids, none

373 of the ash residues showed a color pattern close to white or light gray. Thus, the results

374 here obtained agreed with St. John (30), who stated that neither macroscopic observations

375 nor analyst's judgment for carbon presence in crude ash are useful in determining the

376 optimal ashing methodology.

377 Precision in our study was represented by repeatability, which is also known as

378 within-laboratory variation (24). Repeatability is based on random residual variance, and

379 lower values refer to a more reliable procedure for feed evaluation (34). Repeatability

380 limits were proposed and calculated based on the expected reproducibility and all method

381 variations exhibited repeatability within tolerable limits. However, the use of liquid

382 ashing aids improved repeatability. Such a pattern reinforces our previous discussion.

383 Besides overestimating crude ash, the organic matter retained in the bottom layer of ash

384 residues seems to be variable among test portions. Thus, the action of the liquid ashing

385 aids also improves the precision of the procedures by decreasing random variation among

386 replicates.

387 During the routine of feed analysis, an ideal method must also be able to allow

388 adequate discrimination among and within feed types. That characteristic is expressed

389 here by the relative standard deviations among feed types and study materials. Unlike

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390 repeatability, it is desirable that those relative standard deviations be maximized under

391 any given evaluation. Hence, the capability to discriminate feeds with different

392 characteristics, as well as to cluster similar feeds would be surely guaranteed. Regarding

393 those requisites, all methods evaluated herein performed similarly.

394

Conclusions

396   The method of obtaining residues on ignition in animal feeds based on the

397 binomial 550°C × 3 h does not have sufficient robustness and may overestimate crude

398 ash in some feeds. Adjustments in either ignition time or temperature appear to improve

399 crude ash test results, but the best results are obtained using liquid ashing aids between

400 two ignition cycles. The recommended method is based on the use of 550°C and two 3 h

401 ignition cycles with water added to the ash residue between cycles.

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Acknowledgments

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483

484 **Table 1. Least square means for the residue on ignition (% of dry matter) in different feed types according to the crude ash method**

		Method[a]							
	Temperature (°C)	550	600	550	550	550	550		
	Time (h)	3	3	6	3+3	3+3	3+3		
Feed type	Ashing aid	-	-	-	Fresh Air	Air+H_2O	Air+H_2O_2	SEM	P value
Corn grain		1.18	1.18	1.17	1.13	1.15	1.13		0.689
DDG		3.06	3.00	3.11	3.03	3.04	3.04		0.230
Soybean meal		6.67a	6.49b	6.49b	6.40c	6.33c	6.32c		<0.001
Wheat bran		6.48	6.49	6.44	6.43	6.47	6.45	0.645	0.679
Grass hay		5.39	5.42	5.41	5.41	5.33	5.35		0.244
Corn silage		4.71a	4.72a	4.70a	4.67a	4.59b	4.57b		<0.001
Fresh sugarcane		2.25	2.15	2.22	2.22	2.17	2.20		0.252
Sugarcane silage		4.20ab	4.24a	4.10c	4.18ab	4.12bc	4.11bc		0.003
Overall		4.24a	4.21b	4.20b	4.18b	4.15c	4.15c	0.228	<0.001

485 [a] Means in a row followed by different letters differ at P<0.05.

21

486 **Table 2. Descriptive statistics of random variabilities for the residue on ignition according to the crude ash method**

Item		Method					
	Temperature (°C)	550	600	550	550	550	550
	Time (h)	3	3	6	3+3	3+3	3+3
	Ashing aid	-	-	-	Fresh Air	Air+H_2O	Air+H_2O_2
Relative standard deviations (%)							
Among feed types		43.9	44.0	43.4	43.6	43.6	43.6
Among study materials		26.5	26.4	27.1	26.6	26.6	26.5
Repeatability (r)		2.13	2.73	2.80	2.12	1.50	1.24
Expected reproducibility (%)		3.21	3.22	3.22	3.22	3.22	3.22
Expected limits for r (%)[a]							
Conventional		1.61-2.15	1.61-2.16	1.61-2.16	1.61-2.16	1.61-2.16	1.61-2.16
Maximum tolerable		0.80-4.31	0.80-4.31	0.80-4.31	0.80-4.31	0.81-4.32	0.81-4.32

487 [a] See equations (7), (8), and (9) for more details.

488

22

489 **Table 3. Color pattern of the residue on ignition in different feed types according to the crude ash method**

Feed type[a]		Method					
	Temperature (°C)	550	600	550	550	550	550
	Time (h)	3	3	6	3+3	3+3	3+3
	Ashing aid	-	-	-	Fresh Air	Air+H_2O	Air+H_2O_2
Corn grain	1	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA
DDG	1	(160,158,156)	(156, 154, 153)	(172, 170, 168)	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA
Soybean meal	1	(164, 151, 134)	(205, 181, 154)	(185, 169, 147)	(210, 188, 159)	(213, 188, 160)	(213, 188, 160)
	2	(161, 150, 133)	(211, 185, 156)	(186, 170, 147)	(200, 178, 151)	(219, 194, 167)	(213, 188, 160)
	3	(174, 160, 141)	(216, 190, 162)	(201, 181, 155)	(215, 190, 160)	(218, 191, 165)	(211, 187, 161)
Wheat bran	1	(110, 104, 100)	(119, 117, 115)	(118, 112, 108)	(122, 177, 112)	(114, 111, 108)	(122, 118, 113)
	2	(114, 108, 103)	(120, 117, 116)	(123, 118, 113)	(128, 123, 119)	(118, 115, 113)	(151, 147, 142)
	3	(130, 125, 122)	(164, 160, 157)	(150, 146, 143)	(161, 157, 153)	(166, 159, 154)	(177, 170, 165)
Grass hay	1	(141, 134, 127)	(154, 145, 136)	(141, 133, 123)	(149, 143, 133)	(161, 150, 138)	(165, 154, 142)
	2	(145, 144, 142)	(161, 157, 152)	(152, 149, 148)	(158, 155, 154)	(157, 153, 149)	(170, 165, 160)
	3	(148, 144, 141)	(160, 154, 148	(156, 152, 150)	(155, 151, 149)	(143, 138, 134)	(155, 149, 144)
Corn Silage	1	(125, 119,109)	(139, 130, 118)	(133, 125, 115)	(129, 121, 110)	(145, 130, 112)	(141, 126, 109)
	2	(142, 143, 145)	(152, 151, 150)	(144, 145, 146)	(152, 152, 153)	(126, 122, 120)	(143, 140,138)
	3	(112, 117, 122)	(27, 131, 135)	(118, 122, 128)	(117, 122, 128)	(135, 134, 135)	(119, 120, 122)
Fresh sugarcane	1	(137, 136,135)	(142, 141, 141)	(143, 141, 141)	(144, 143, 144)	(132, 128, 126)	NA
	2	(150, 147, 145)	(162, 157, 152)	(161, 157, 153)	NA	NA	NA
	3	(152, 146, 139)	(163, 153, 140)	(149, 143, 134)	(160, 152, 142)	NA	NA
Sugarcane silage	1	(160, 155, 149)	(175, 166, 155)	(167, 161, 154)	(172, 165, 155)	NA	NA
	2	(107 ,105, 105)	(122, 120, 119)	(118, 116, 116)	(108, 107, 107)	(118, 113, 109)	(122, 116, 112)
	3	(121, 113, 104)	(139, 128, 118)	(136, 126, 116)	(128, 118, 108)	(133, 118,104)	(136, 121, 107)

490 [a] The numbers 1 to 3 indicate the different study materials (i.e., field samples) evaluated within each feed type.

491   NA: not available. The residue on ignition was insufficient for the color evaluation using the colorimeter.
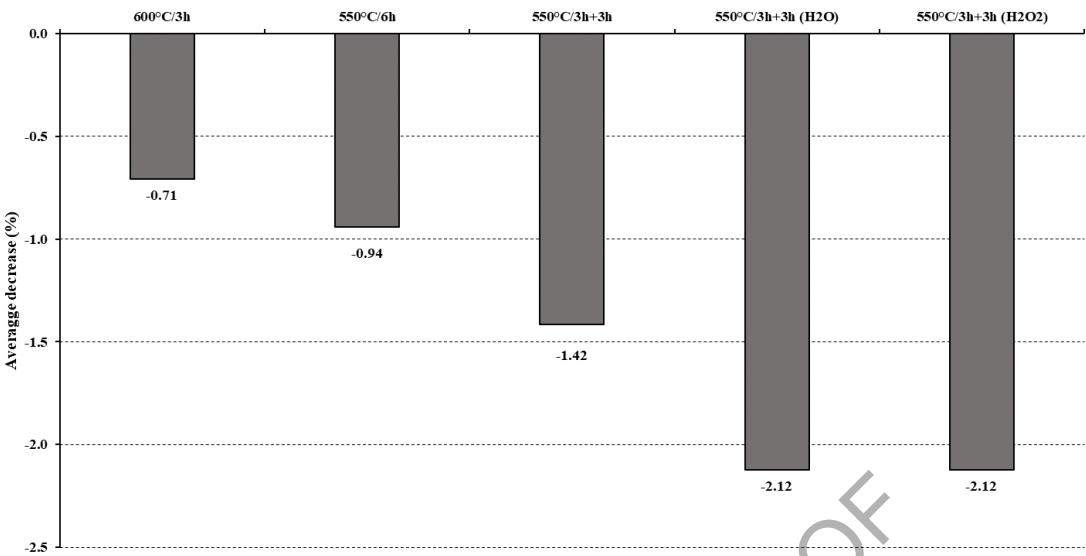
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494 **Figure 1. Average percentage decrease in residue on ignition according to the crude**

495 **ash methods in relation to the control method (i.e., 550°C × 3 h).**